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In vitro study of the cytotoxicities of two mixed-ligand oxovanadium complexes on human hepatoma cells

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The cytotoxicities of two oxovanadium complexes, VOI [VO(satsc)(phen)] (satsc = salicylaldehyde thiosemicarbazone, phen = 1,10-phenanthroline) and VOII [VO(3,5-dibrsatsc)(phen)](3,5-dibrsatsc = 3,5-dibromosalicylaldehyde thiosemicarbazone), were studied by performing MTT assays on human hepatoma cell lines BEL-7402, HUH-7 and HepG2. The results showed that both the VOI and VOII complexes possess significant anti-proliferative effects. In addition, the anti-proliferative mechanism of the complexes was analyzed by cell cycle analysis and an apoptosis assay and by detecting the mitochondrial membrane potential ($\Delta\Psi$m). The experimental results showed that the complexes can cause a G0/G1 phase cell cycle arrest and can significantly decrease $\Delta\Psi$m, causing depolarization of the mitochondrial membrane. Notably, the two complexes induced apoptosis in BEL-7402 cells and displayed typical morphological apoptotic characteristics. The cytotoxicities of the VOII complex are significantly stronger than that of the VOI complex, suggesting that the cytotoxic effects of oxovanadium complexes may be associated with the electronic effects of the complexes.

1. Introduction

Cancer is currently one of the major causes of mortality in humans throughout the world, and it is still the most life-threatening and challenging disease (Jemal et al. 2009). Hepatocellular carcinoma (HCC) is the sixth most common cancer globally in terms of the number of cases (626,000, or 5.7% of new cancer cases every year). However, because of the disease's poor prognosis, the number of deaths from HCC every year is nearly equal to the number of cases (598,000). Although ranked sixth among the common cancers, HCC causes third malignancy-related mortality with survival rates of 3% to 5% according to cancer registries in the United States and other countries (Parkin et al. 2005). Half of these cases and deaths were estimated to occur in China (Ferlay et al. 2010). Currently, surgical resection and transplantation are the only proven cures for HCC. Unfortunately, most patients who undergo hepatectomy are reported to develop new tumors in the residual liver (Carr 2004; Song et al. 2004). As available treatments are rarely effective, patients' prognosis is poor (Kern et al. 2002; Ying et al. 2008). Thus, new and more effective therapeutic agents and treatment regimens for HCC are needed (Zhang et al. 2011). Metallo-drugs are the most promising target in the search for new therapeutic tools against cancer (Ying et al. 2008; Zhang et al. 2011; Hirao 1997; Wever and Kustin 1990). Vanadium is a biologically essential trace element, and vanadium complexes have been reported to be involved in a wide range of biological activities, including enzyme inhibition, antitumor activity and insulin mimesis (Crans 1994; Heyliger et al. 1985; Crans et al. 1997; Yuen et al. 1997; Prasad et al. 2010; Sasmal et al. 2010). Oxovanadium complexes, particularly those with phenanthroline ligands, exhibit potent cytotoxic activity, DNA binding activity and DNA-photocleavage activity (Alvin 2011; Sun et al. 2010; Subhashree et al. 2012; Benítez et al. 2011). In addition, Schiff bases and their first-row transition metal complexes exhibit fungicidal, bactericidal, antiviral, and antitubercular activity. Vanadium-Schiff base complexes are receiving considerable attention (Benítez et al. 2011).

In a previous work, we synthesized and characterized VOI [VO(satsc)(phen)] (satsc = salicylaldehyde thiosemicarbazone, phen = phenanthroline) and VOII [VO(3,5-dibrsatsc)(phen)](3,5-dibrsatsc = 3,5-dibromosalicylaldehyde thiosemicarbazone), which are two oxovanadium complexes with a multidentate Schiff base ligand and 1,10-phenanthroline (Lu et al. 2011; Du et al. 2010), and studied their DNA-binding properties. We found that these complexes bind to CT-DNA *via* intercalation modes. Moreover, it has been proposed that compounds that efficiently interact with DNA by an intercalative mode may also show antitumor activities (Benítez et al. 2011). We have also reported that four oxovanadium (IV) complexes are highly cytotoxic against both a myeloma cell line (Ag8.653) and a glioma cell line (U251) (Lu et al. 2012). In continuing our research on this subject, we report here the cytotoxicities of the VOI and VOII complexes on proliferation of the human hepatoma cell lines BEL-7402, HUH-7 and HepG2 and the biological activities on cell cycle progression

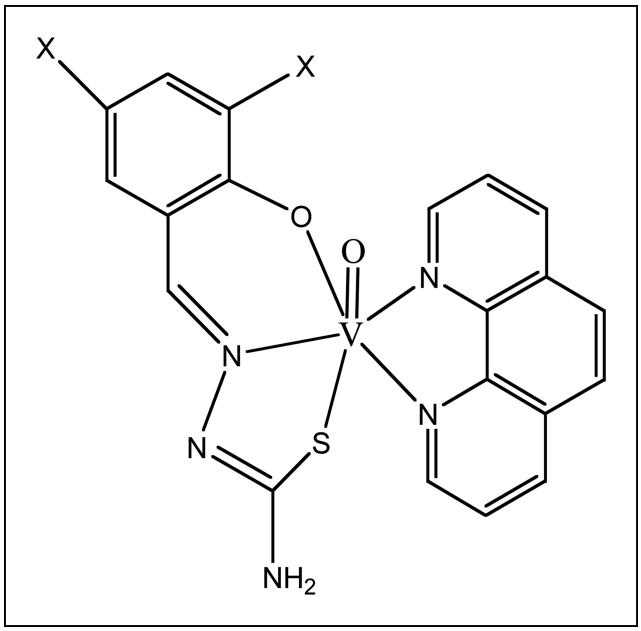

Fig. 1: Structures of VOI [VO(satsc)(phen)], X = H and VOII [VO(3,5-dibrsatsc)(phen)], X = Br.

and apoptosis of the BEL-7402 cells using various techniques. The oxovanadium complexes used in this study are shown in Fig. 1.

2. Investigations and results

2.1. Anti-proliferative effects of the VOI and VOII complexes on the BEL-7402, HUH-7 and HepG2 hepatoma cells

The VOI and VOII complexes were evaluated for their ability to inhibit the growth of the BEL-7402, HUH-7 and HepG2 human hepatoma cell lines using the MTT assay. Inhibition was calculated as cell viability relative to control cells without the VOI and VOII complex treatment 5-Fluorouracil (5-FU, 30 μM) was used as the positive control.

Cell viability after treatment with the VOI and VOII complexes for 24 h, 48 h or 72 h on the selected three cell lines is shown in Figs. 2–4, and the IC_{50} values are summarized in Table 1. The MTT assay results indicate that the inhibition of cell growth increases significantly with the up-regulation in the concentration of the VOI and VOII complexes in a dose- and time-dependent manner. The cytotoxic effects of the VOII complex are greater than that of the VOI complex, thus the VOII complex may confer greater anti-proliferative properties to cancer cells.

2.2. Cell cycle analysis

To further define the biological mechanism of the anti-proliferative effects of the two compounds on cancer cells, cell cycle distribution was analyzed by flow cytometry using PI staining (Xu et al. 2010; Johnstone et al. 2002; Frankfurt and Krishan 2003). The BEL-7402 cells were treated with 30 μM, 60 μM or 120 μM of the VOI or VOII complex for 48 h. The results showed that the number of cells in the G0/G1 phase was significantly increased and the number of cells in the S and G2/M phases was significantly decreased after the BEL-7402 cells were exposed to the VOI and VOII complexes in comparison to untreated cells (Figs. 5,6 Table 2). These results indicate that the VOI and VOII complexes inhibit growth of the BEL-7402 cells by inducing a block at the G0/G1 phase of the cell cycle; in addition, the cell cycle arrest induced by the VOII complex is stronger than that of the VOI complex.

2.3. Induction of apoptosis as evidenced by Hoechst 33342 staining

To determine whether the inhibition of cell growth in the BEL-7402 cells induced by the VOI and VOII complexes was associated with apoptosis, the prevalence of apoptosis was determined using Hoechst 33342 staining. Figs. 7 and 8 shows representative staining fluorescence photomicrographs of the cultured BEL-7402 cells with or without the VOI and VOII complexes. Compared to the control cells, the BEL-7402 cells exhibited typical apoptotic features after treatment with the VOI and VOII complexes for 48 h, including cellular morphological changes, membrane blebbing, the formation of apoptotic bodies and the condensation of the chromatin (brightly stained).

2.4. Apoptosis assessment by Annexin V-FITC/PI assay

To further investigate the induced apoptosis effects of the VOI and VOII complexes, Annexin V-FITC/PI staining was performed to determine early apoptotic and necrotic cells, with 5-fluorouracil (5-FU, 30 μM) used as the positive control. As shown in Figs. 9 and 10, the percentage of Annexin V-FITC-stained early apoptotic cells increased with increasing

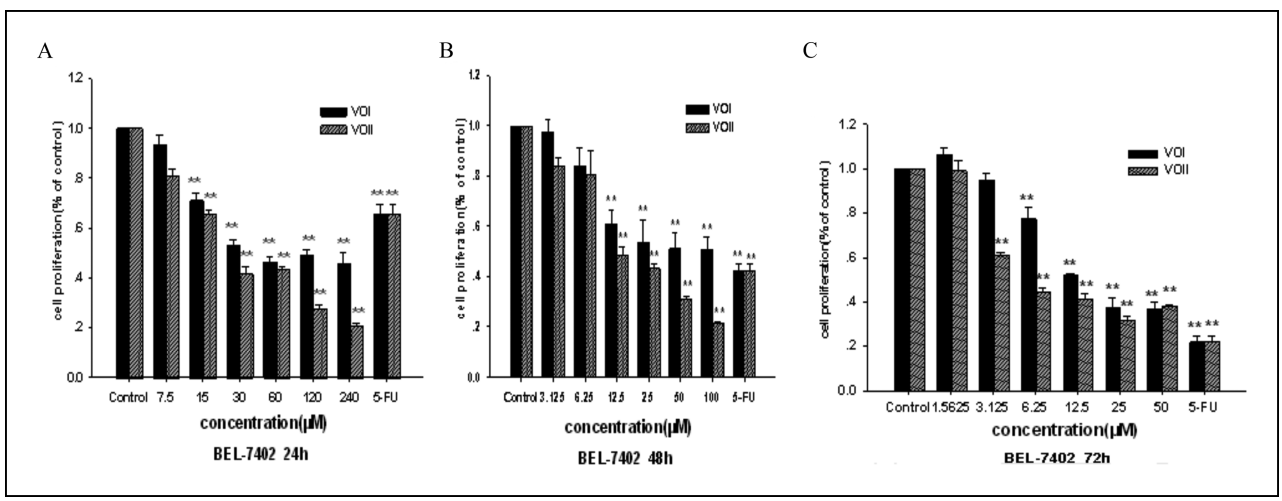

Fig. 2: The anti-proliferative activity of the VOI and VOII complexes on BEL-7402 cells as detected by an MTT assay after 24 h (A), 48 h (B) or 72 h (C) of treatment.

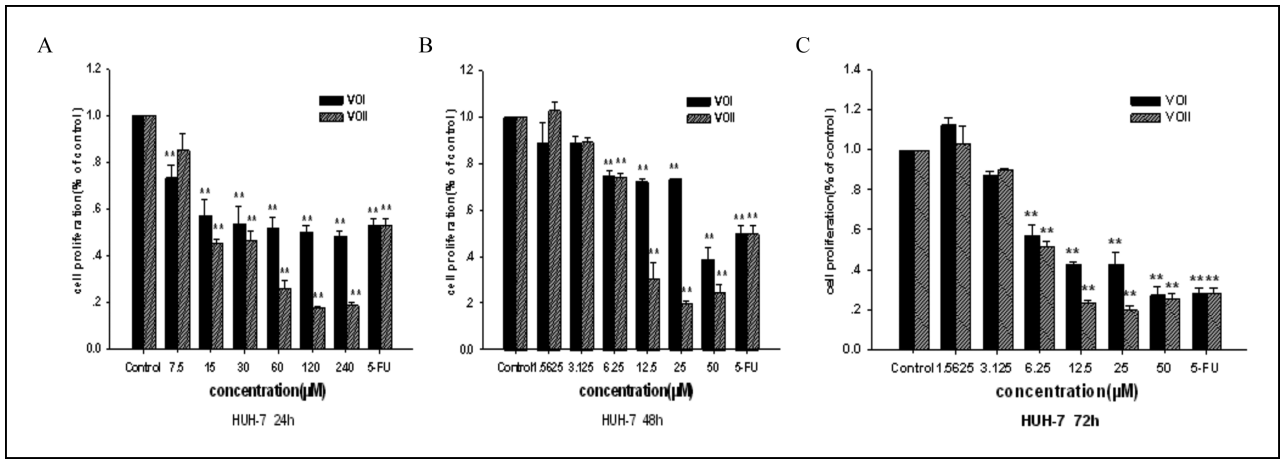

Fig. 3: The anti-proliferative activity of the VOI and VOII complexes on HUH-7 cells as detected. by an MTT assay after 24 h (A), 48 h (B) or 72 h (C) of treatment.

Table 1: Comparison of IC_{50} values obtained from the MTT assay on the BEL-7402, HUH-7 and HepG2 hepatoma cell lines after treatment for 24 h, 48 h or 72 h using the VOI and VOII complexes

Cell line complex		IC_{50} values obtained from the MTT assay (μM)		
		24 h	48 h	72 h
BEL-7402	VOI	**100.53 ± 4.95** [a]	**55.16 ± 3.89** [b]	20.18 ± 2.63 [c]
	VOII.	**35.10 ± 3.07** [a]	**19.46 ± 2.14** [b]	10.89 ± 2.06 [c]
HUH-7	VOI	**112.08 ± 4.71** [a]	47.93 ± 4.22 [b]	12.30 ± 1.38 [c]
	VOII.	**24.12 ± 2. 78** [a]	11.65 ± 1.85 [b]	8.93 ± 1.44 [c]
HepG2	VOI	8.25 ± 1.13 [a]	6.80 ± 0.76 [a]	3.94 ± 0.85 [b]
	VOII.	7.35 ± 1.40 [a]	1.68 ± 0.41 [b]	3.24 ± 0.52 [b]

Data are the mean ± SD of at least three independent experiments.*Between two differ letter $P < 0.05$, between the same letter $P > 0.05$.

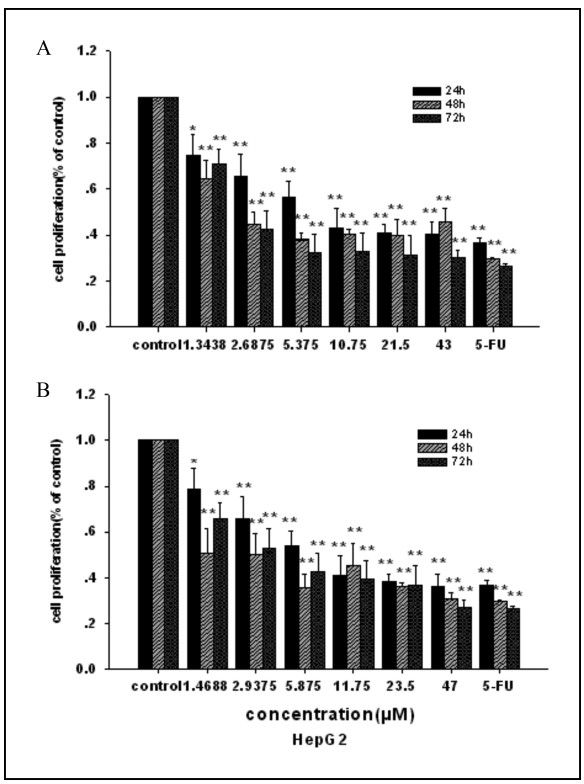

Fig. 4: The anti-proliferative activity of the VOI (A) and VOII (B) complexes on the HepG2 cells as detected by the MTT assay after 24 h, 48 h or 72 h of treatment.

concentrations (30 μM, 60 μM, and 120 μM) of the VOI and VOII complexes. It is interesting to note that in cells treated with different concentrations of the VOI and VOII complexes for 48 h, the percentage of early apoptotic cells induced by the VOII complex was significantly higher than that induced by the VOI complex. These results suggest that the VOI and VOII complexes induce proliferative suppression of the BEL-7402 cells *via* the induction of apoptosis and the induced apoptosis effect of the VOII complex is stronger than that of the VOI complex.

2.5. Loss of mitochondrial membrane potential (△Ψm)

To evaluate the mitochondria during VOI- and VOII-induced apoptosis, we investigated the ability of the VOI and VOII complexes to induce alterations in the mitochondrial membrane potential. As shown in Table 3, the mean value of the green fluorescence intensity, as measured by flow cytometry on the BEL-7402 cells after treatment for 48 h, was gradually increased with corresponding increases in the VOI and VOII complex concentration (60, 90 and 135 μM). These data show that much of the rhodamine123 from the mitochondria matrix was released into the cytoplasm in a dose-dependent manner, indicating that the VOI and VOII complexes can affect mitochondrial function and cause the depolarization of the mitochondrial membrane, leading to a significantly decreased ΔΨm value. Therefore, the VOI and VOII complexes may induce apoptosis through the mitochondrial pathway.

3. Discussion

VOI and VOII are two oxovanadium complexes with a multidentate Schiff base ligand and a 1,10-phenanthroline (Lu et al. 2011; Du et al. 2010). At present, studies on the cytotoxic effects for tumor cells of Vanadium-Schiff base complexes are receiving considerable attention. Therefore, we systematically

Table 2: Cell cycle analysis of the BEL-7402 cells treated with the VOI and VOII complexes for 48 h

Concentration (μM)		Relative proportion of the different phases of the cell cycle (%)		
		G0/G1	S	G2/M
Control		62.57 ± 1.53	10.82 ± 1.50	21.67 ± 2.60
VOI	30	65.88 ± 5.22	14.55 ± 3.61	16.84 ± 5.25
	60	75.67 ± 1.63**	9.13 ± 2.84	11.07 ± 1.76**
	120	76.30 ± 0.46**	9.04 ± 2.48	9.78 ± 2.11**
VOII.	30	78.43 ± 2.15**	7.73 ± 1.16*	8.21 ± 1.73**
	60	80.64 ± 3.59**	7.42 ± 1.38*	7.50 ± 2.31**
	120	78.97 ± 1.95**	7.20 ± 1.68*	7.33 ± 2.53**

Data are the mean ± SD of at least three independent experiments. * For $P < 0.05$; ** For $P < 0.01$. * For $P < 0.05$ versus the control, the difference was significant. ** For $P < 0.01$ versus the control, the difference was markedly significant.

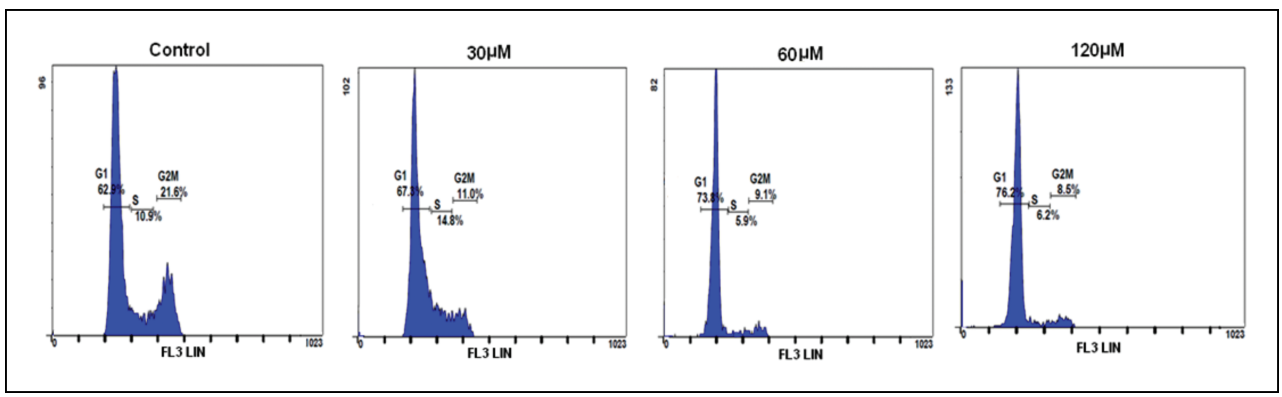

Fig. 5: The DNA content and cell cycle analysis of the BEL-7402 cells after treatment with the VOI complex. The BEL-7402 cells were cultured with either 0.1% DMSO (control) or with 30 μM, 60 μM or 120 μM of the VOI complex for 48 h. The percentage of non-apoptotic cells within each cell cycle was determined by flow cytometry.

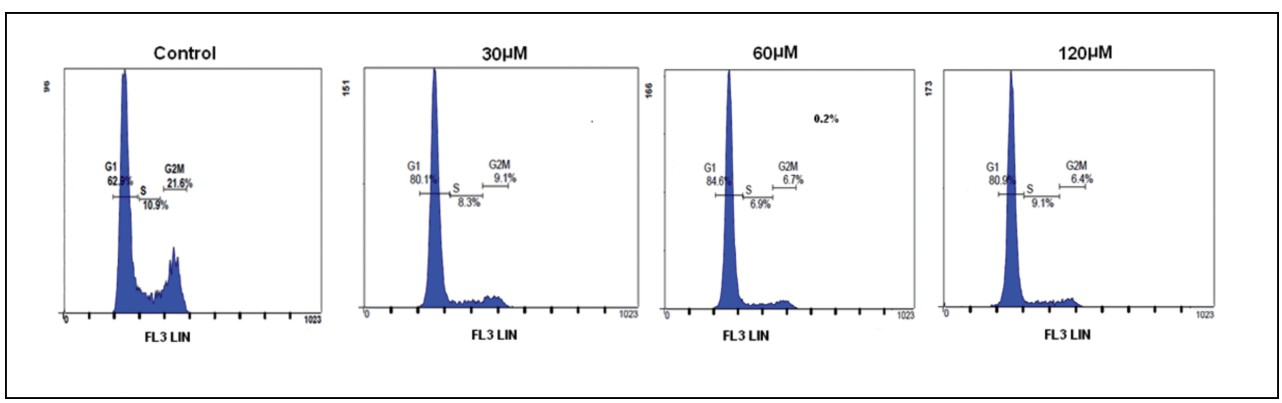

Fig. 6: The DNA content and cell cycle analysis of the BEL-7402 cells after treatment with the VOII complex. The BEL-7402 cells were cultured with either 0.1% DMSO (control) or with 30 μM, 60 μM or 120 μM of the VOII complex for 48 h. The percentage of non-apoptotic cells within each cell cycle was determined by flow cytometry.

investigated the anti-proliferative biological activities of the VOI and VOII complexes *in vitro*. From the results of the MTT assay, we found that both of the oxovanadium complexes exhibit an anti-proliferative effect on the human hepatoma cells BEL-7402, HUH-7 and HepG2 in a time- and dose-dependent manner directly related to the concentration of the VOI and VOII com-

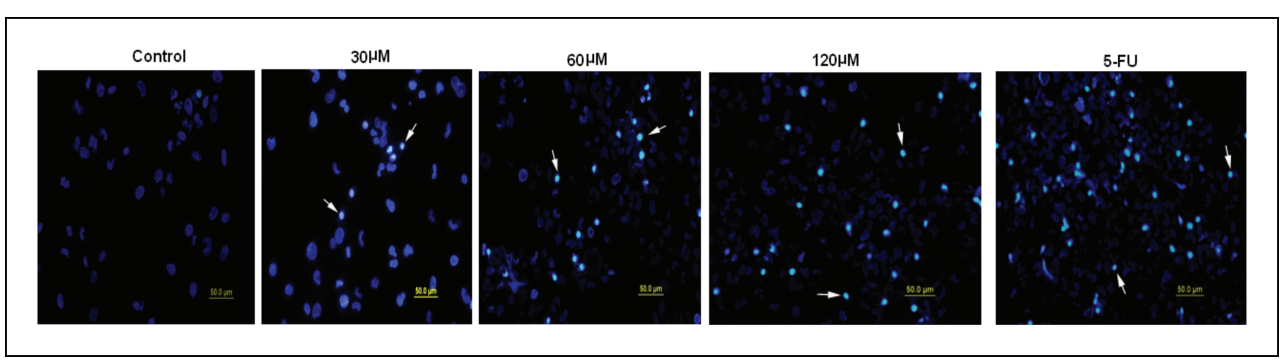

Fig. 7: The effects of the VOI complex on the morphology of the Bel-7402 cells as assayed by Hoechst 33342 staining. After treatment with the VOI complex for 48 h, apoptotic cells were detected by Hoechst 33342 staining and examined by fluorescence microscopy, model Olympus BX51 (original magnification 400×).

Table 3: Depolarization of the mitochondrial membrane potential of the BEL-7402 cells treated with the VOI and VOII complexes for 48 h

Concentration (μM)	% of control (mean value of green fluorescence intensity)	
	VOI	VOII
Control	100.00 ± 1.40	102.48 ± 2.52
60	111.79 ± 4.56	108.57 ± 6.46
90	136.95 ± 2.48**	177.66 ± 5.45**
135	157.84 ± 9.42**	214.76 ± 7.76**

Data are the mean ± SD of at least three independent experiments. * For $P < 0.05$; ** For $P < 0.01$. * For $P < 0.05$ versus the control, the difference was significant. ** For $P < 0.01$ versus the control, the difference was markedly significant.

plexes. 5-Fluorouracil (5-FU) was used as a positive control because it has been used extensively as an efficient anticancer drug in clinical trials (Xu et al. 2010; Johnstone et al. 2002; Frankfurt and Krishan 2003). The IC_{50} values of the VOII complex on the BEL-7402, HUH-7 and HepG2 cells after treatment for 24 h, 48 h or 72 h were less than that of the VOI complex (Figs. 2–4, Table 1), suggesting that the VOII complex possesses a more potent inhibitory effect against the cancer cells. This difference may be attributed to the introduction of two bromides on the 3- and 5-positions of the aromatic chromophore of salicylaldehyde thiosemicarbazone, which would imply that the electronic effect of salicylaldehyde thiosemicarbazone is one of the factors in determining the anti-cancer abilities of oxovanadium complexes. These data are consistent with the VOI and VOII complexes binding abilities with CT DNA (Lu et al. 2012), indicating that the cytotoxic abilities of these two oxovanadium complexes may be closely related to their DNA binding mode.

To further determine the biological mechanisms of VOI- and VOII-induced growth inhibition, the effects on the nuclear

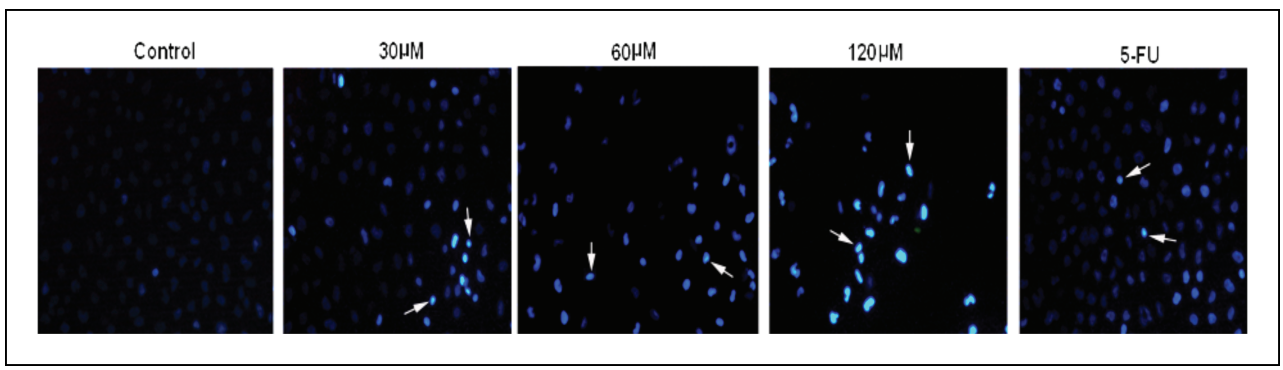

Fig. 8: The effects of the VOII complex on the morphology of Bel-7402 cells as assayed by Hoechst 33342 staining. After treatment with the VOII complex for 48 h, apoptotic cells were detected by Hoechst 33342 staining and examined by fluorescence microscopy model DMI 3000B (original magnification 400×).

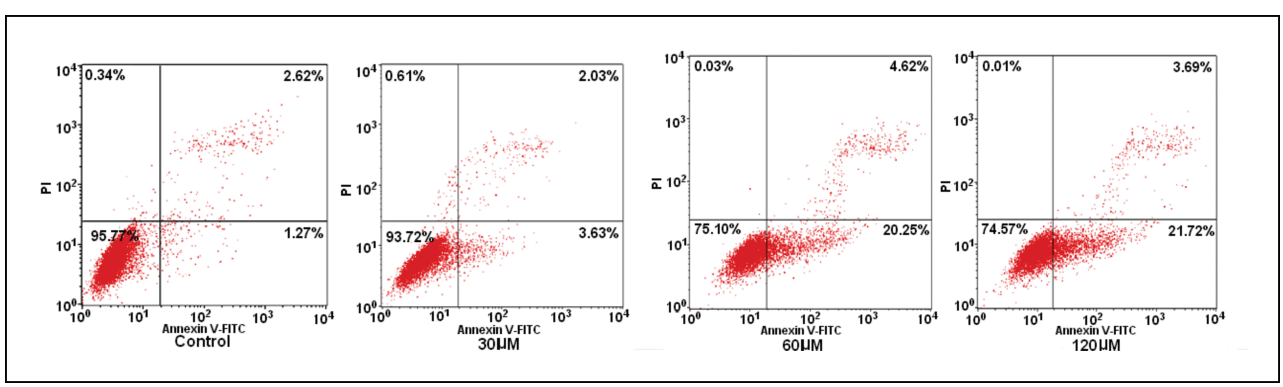

Fig. 9: The distribution map of cell apoptosis. The BEL-7402 cells were incubated with different concentrations of the VOI (30, 60 or 120 μM) complex for 48 h, subjected to Annexin V-FITC/PI staining, and analyzed by flow cytometry.

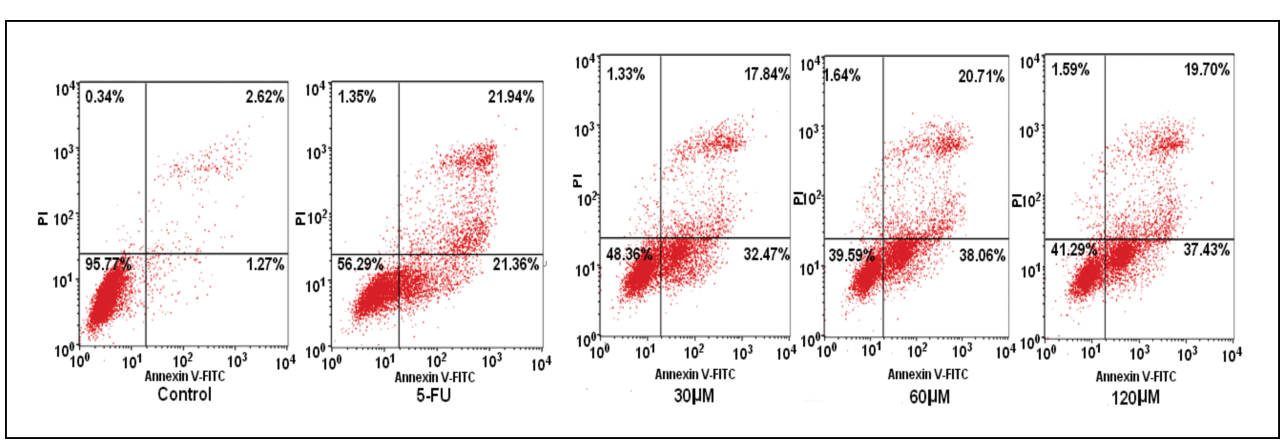

Fig. 10: The distribution map of cell apoptosis. The BEL-7402 cells were incubated with different concentrations of the VOII complex (30, 60 or 120 μM) for 48 h, subjected to Annexin V-FITC/PI staining, and analyzed by flow cytometry.

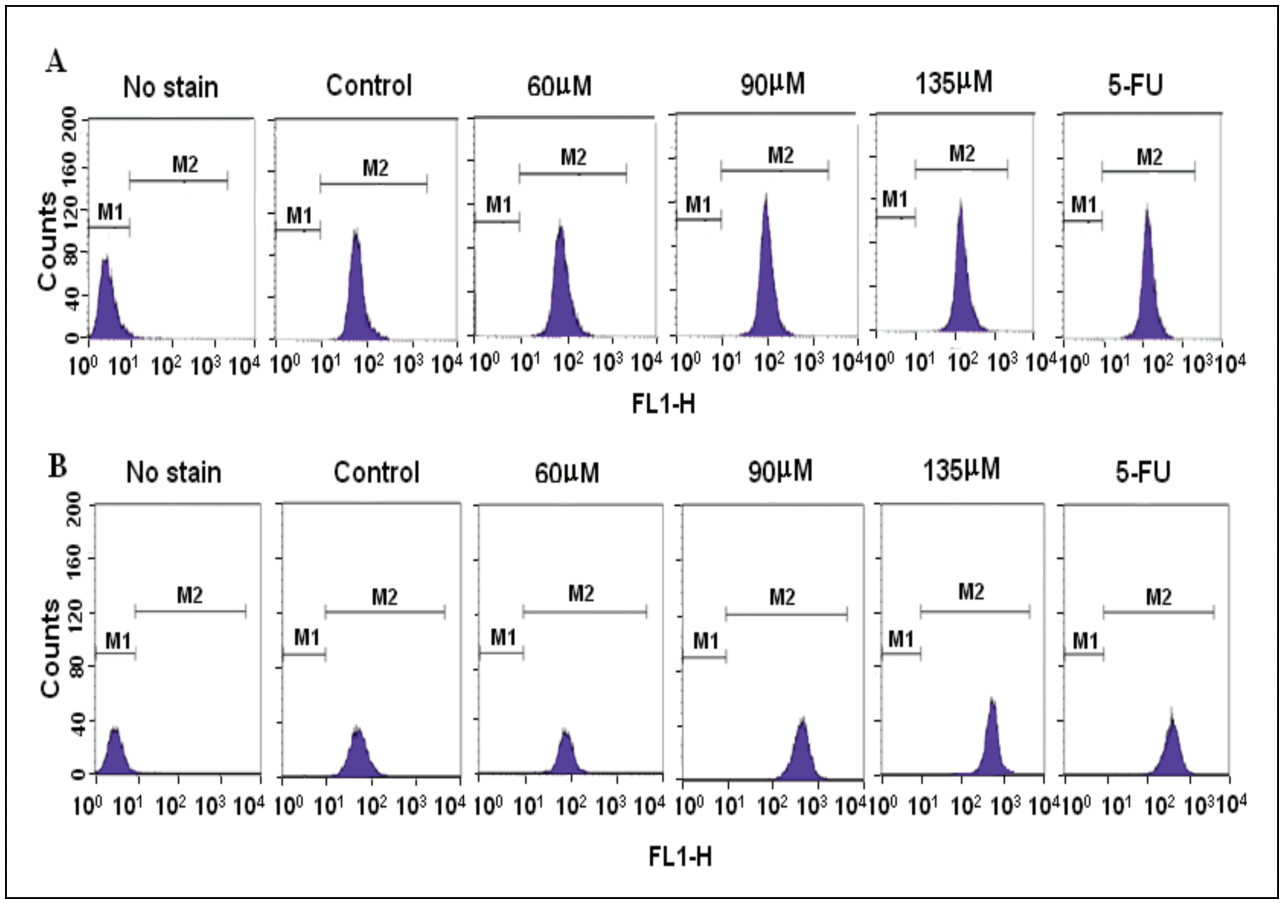

Fig. 11: The distribution map of mitochondrial membrane potential. The BEL-7402 cells were incubated with different concentrations of the VOI (A) and VOII (B) complexes (60, 90 or 135 μM) for 48 h, subjected to Rhodamine123 staining, and analyzed by flow cytometry. Note: M1 represents negative, non-fluorescently marked cells; M2 represents positive, fluorescently marked cells; the abscissa represents the size of the green fluorescence intensity; the ordinate represents cell counts.

changes were investigated using flow cytometry. As shown in Table 2, pretreatments with the VOI and VOII complexes at 30, 60, or 120 μM significantly increased the G0/G1 phase distribution and decreased the S and G2/M phase distribution in a dose-dependent manner. Therefore, the oxovanadium complexes VOI and VOII inhibited the growth of the BEL-7402 cells by inducing a block in the G0/G1 phase of the cell cycle, and the arrested effect of the VOII complex was stronger than that of the VOI complex. This result is consistent with the anti-proliferative effects of the VOI and VOII complexes. The experimental results of the cell cycle analysis thus provide strong evidence that the cytotoxic effects of these two complexes are closely associated with the electronic effects of the substituents introduced on the aromatic chromophore of the ligands. These results also suggest that the two oxovanadium complexes induced proliferative suppression of the BEL-7402 cells, possibly *via* the induction of apoptosis (Xu et al. 2010; Johnstone et al. 2002; Frankfurt and Krishan 2003; Hu and Kavanagh 2003).

Apoptosis is an important continuous process of destructing undesirable cells during development or homeostasis in multicellular organisms. This process is characterized by distinct morphological changes that include membrane blebbing, cell shrinkage, dissipation of the mitochondrial membrane potential ($\Delta\Psi$m), chromatin condensation and DNA fragmentation (Frankfurt and Krishan 2003; Hu and Kavanagh 2003; Ly et al. 2003; Kaufmann and Hengartner 2001). It is also known that cancer is caused by the disruption of cellular homeostasis between cell death and cell proliferation (Kaufmann and Hengartner 2001; Thompson 1995; Martin and Green 1995), and compounds that can induce apoptosis are considered to be potential anticancer drugs (Ying et al. 2008; Johnstone et al. 2002; Frankfurt and Krishan 2003). As seen in Figs. 7 and 8, the BEL-7402 cells treated with the VOI and VOII complexes showed significant characteristics of apoptotic cells. In addition, it is known that phosphatidylserine (PS) externalization is an early feature of apoptosis and can be detected by the binding of Annexin V to PS on the cell surface (Martin and Green 1995; Vermes 1995; Liu et al. 2010; Kostova 2009). The assessment of apoptosis by the Annexin V-FITC/PI assay is well recognized and accurate. According to the results of the Annexin V-FITC/PI assay, both the VOI and VOII complexes induced proliferative suppression of the BEL-7402 cells via the induction of apoptosis, and the induced apoptosis effect of the VOII complex was stronger than that of the VOI complex. The experimental results of the apoptosis assessment provide further evidence that the anti-proliferative effects of these two complexes are associated with the electronic effects of the complexes. The disruption of the $\Delta\Psi$m is one of the earliest intracellular events that occur following the induction of apoptosis (Thompson et al. 1999). To evaluate the mitochondria during VOI- and VOII-induced apoptosis, the ability of the VOI and VOII complexes to induce alterations in the mitochondrial potential was investigated. The results showed that the mean value of the green fluorescence intensity, as measured by flow cytometry on the BEL-7402 cells after treatment for 48 h, was significantly increased (Fig. 11, Table 3). This finding indicates that much of the rhodamine123 from the mitochondria matrix was released into the cytoplasm as the concentration of the VOI and VOII complexes increased. Moreover, the two complexes decreased the $\Delta\Psi$m significantly, causing the depolarization of the mitochondrial

membrane. Therefore, the loss of transmembrane potential was involved in VOI- and VOII-induced apoptosis. However, further research on the mechanisms of apoptosis induction is necessary.

In conclusion, this study shows that both the VOI and VOII complexes possess significant anti-proliferative effects on cancer cells. Moreover, they can cause cell cycle to arrest at the G0/G1 phase, they can induce apoptosis, and they can significantly decrease the mitochondrial membrane potential. In addition, the VOII complex exhibits greater anti-proliferative biological efficiency than the VOI complex, suggesting that the cytotoxic effects of the oxidovanadium complexes may be associated with the complexes' electronic effects.

4. Experimental

4.1. Materials

The VOI and VOII complexes were provided by Dr. Lu (Chemistry Department of the School of Pharmacy, Guangdong Pharmaceutical University)(Lu et al. 2011; Du et al. 2010; Lu et al. 2012). The purities of the VOI and VOII complexes used in the experiments were > 95%, as determined by HPLC. The complexes were dissolved in DMSO and added to the experimental media to yield the final concentrations.

DMSO and $CHCl_3$ were purchased from Aldrich (USA). Other chemicals and reagents of analytical grade were obtained commercially without further purification unless noted.

BEL-7402, HUH-7 and HepG2 cell lines were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RPMI- 1640 medium was purchased from Hyclone (Logan, USA). Trypsin, fetal calf serum and an Annexin V-FITC/PI apoptosis detection kit were purchased from GIBCO (USA). The Hoechst 33342 staining solution was purchased from the Beyotime Institute of Biotechnology (China). MTT and rhodamine123 were purchased from Sigma (USA).

4.2. Cell culture

The cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. The culture was maintained at 37 °C, with a gas mixture of 5% CO_2/95% air. The medium was changed every two days, and the cells were subcultured every three days.

4.3. Cell viability assays

Cell viability was determined using the MTT assay. The cells were collected and resuspended in RPMI-1640 medium at 4×10^4 cells/ml, and 100 μl aliquots were then added to each well of 96-well flat-bottomed microtiter plates, followed by the addition of 100 μl of the VOI and VOII complexes. Three replicate wells were used for each data point in the experiments. After incubation for the indicated intervals, 20 μl of MTT (5 mg/ml in PBS) solution was added to each well, and the plates were then incubated for 4 h at 37 °C. The medium with MTT was removed from the wells. The intracellular formazan crystals were dissolved by the addition of 150 μl of DMSO to each well, and the plates were shaken for 10 min. The absorbance was read at 490 nm with a microplate reader (Model 680 Microplate Reader, BIO-RAD, USA). The survival percentage was calculated as a fraction of the negative control (0.1%DMSO). The half-maximal inhibitory concentration (IC_{50}) was obtained from the dose-response curve with original 6.0 software.

4.4. Cell cycle analysis

The cell cycle control of the treated cancer cells was analyzed. Using standard methods, the DNA of the cells was stained with PI, and the proportion of non-apoptotic cells in different phases of the cell cycle was recorded. The cancer cells were treated with the VOI and VOII complexes, harvested by centrifugation at 1000 × g for 5 min, and washed with ice-cold PBS. The collected cells were fixed overnight with cold 70% ethanol and then stained with a PI solution consisting of 50 μg/ml PI and 10 μg/ml RNase. After a 10 min incubation at room temperature in the dark, fluorescence-activated cells were sorted in a FACScan flow cytometer (BD FACSCalibur TM, USA) using CellQuest 3.0.1 software.

4.5. Fluorescence microscopy of the apoptosis assays

This method was modified from a previous report (Zheng et al. 2011). Briefly, after exposure to the VOI and VOII complexes for 48 h, the BEL-7402 cells were washed twice with PBS and stained with 10 μM of Hoechst 33342 staining solution for 30 min at 37 °C, according to the manufacturer's instructions. Finally, the cells were observed under a fluorescence microscope. In experiments for the VOI complex, the apoptotic cells were examined by fluorescence microscopy, BX51 system of Olympus Company (Japan). In experiments for the VOII complex, the apoptotic cells were examined by fluorescence microscopy, DMI 3000B system of Leica Company (Germany).

4.6. Annexin V-FITC/PI assays of apoptotic cells

The BEL-7402 cells that had been treated with the VOI and VOII complexes for 48 h were analyzed by flow cytometry using a commercially available Annexin V-FITC/PI Apoptosis Detection Kit. After treatment, the cells were harvested and washed twice in ice-cold PBS and resuspended in 500 μl of binding buffer at 1–5 × 10^5 cells/ml. The samples were then incubated with 5 μl of Annexin V-FITC and 5 μl of propidium iodide in the dark for 15 min at room temperature. Finally, the samples were analyzed by flow cytometry (BD FACSCalibur TM, USA) and evaluated based on the percentage of cells that were positive for Annexin V.

4.7. Detection of mitochondrial membrane potential (ΔΨm)

In this study, ΔΨm was measured using rhodamine123, and 5-fluorouracil (5-FU, 50 μM) was used as the positive control. After treatment with the VOI and VOII complexes for 48 h, the BEL-7402 cells were incubated with rhodamine123 (20 μM) 0.2 ml for 30 min at 37 °C and then washed with PBS. The cell pellets were collected by centrifugation (1000 × g, 5 min) and resuspended in 500 μl of PBS. The green fluorescence intensities of rhodamine123 in cells were analyzed in the FL-1 channel of the flow cytometer (BD FACSCalibur TM, USA). This method for detection of ΔΨm was modified from an earlier report (Zhen et al. 2011).

4.8. Statistical analysis

The data were expressed as the mean ± SD from these independent experiments. Statistic analysis was performed using SPSS 13.0 for Windows. Comparisons between the two groups were performed using an unpaired t-test. Multiple comparisons between more than two groups were performed by one-way analysis of variance (ANOVA). Significance was accepted at a P value lower than 0.05.

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